#7

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

TANAKA, Akiko; **JESSIP**, John; and **BRADLEY**, William Guy

Application No.: **09/964,240** Art Unit: **1654**

Filed: **September 26, 2001** Examiner: **TATE, C.**

For: **PINE CONE EXTRACTS AND USES THEREOF** Conf. No.: **1854**

Attorney Docket: **3974.002**

DECLARATION UNDER 37 C.F.R. §1.131

Mail Stop Non-Fee Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Akiko TANAKA, of 10900 Roosevelt Blvd., St.Petersburg, Florida 33716-2308, declare and state as follows:

1. I am a co-inventor of the invention disclosed and claimed in the above-identified application for U.S. Patent, am familiar with the contents of the Office Action mailed in regard to said application on March 24, 2003, and submit this Declaration for the purpose of establishing a date of invention prior to the date of publication of the reference Xin (CN 1279107), which is cited in said Office Action.

2. I am a research virologist possessing a Ph.D. in virology, and was co-founder in 1981 of the Tampa Bay Research Institute in St.Petersburg, Florida, where I am currently a Senior Member and Principal Investigator, and where I have been, since 1995,

its President.

3. Prior to June 1, 1999, as the result of a collaboration between Akiko TANAKA, William Guy BRADLEY, and John JESSIP, (herein collectively "we"), I contributed to conception of the subject invention as disclosed and claimed in the above-captioned application for U.S. Patent.

4. On June 1, 1999, and in part as a result of the collaboration described in paragraph 4, a proposal for research funding naming William Guy BRADLEY, Ph.D., as Principal Investigator, and titled "**Dissecting the Legendary Anti-Tumor Activity of a Pine Complex: Activating the Anti-Tumor Potential of Macrophage and Dendritic Cells,**" was submitted to the **U.S. Army Medical Research and Materiel Command,** and that a copy of this proposal and accompanying letter of submission is appended as Appendix A.

5. Receipt of the proposal of Appendix A was acknowledged by mail in the form of a postcard, a copy of which is appended as Appendix B, which postcard was mailed by the office of Dr. Kenneth A. Bertram, Lt. Col., U.S. Army Medical Corps, to Dr. William G. Bradley, assigning identification number BC990863 to the proposal.

6. The proposal of Appendix A sets forth, as described below, the essence of the invention of the above-captioned application for U.S. patent.

7. I was aware from prior studies that activation of dendritic cells in vitro in the presence of tumor antigen and specific cytokines can induce tumor-specific cytotoxicity when the dendritic cells thus treated are used as a tumor vaccine. See Appendix A at page 8, lines 4-5.

8. In the proposal of Appendix A, we proposed the hypothesis that the host-mediated anti-tumor activity of pinecone extract resides in its ability to alter immune function in favor of a Th1 response, and in its ability to activate dendritic and macrophage cells. See Appendix A at p. 10, lines 3-6.

9. I understood at the time of filing the grant application of Appendix A (June 1, 1999) that the only known function of dendritic cells was to present antigen to T-cells, and that the mature dendritic cells found in lymphoid tissues were generally known to be by far the most potent stimulators of naïve T cells. See Janeway "The Immune System in Health and Disease" 5th Edn., Garland Publishing, at p.307, §8-6, and Appendix A at p.7, ¶¶3-5, et seq.

10. It was my belief and opinion at the time of filing the proposal of Appendix A on June 1, 1999, that an agent which boosted dendritic cell function would also boost antigen presentation to T-cells, which is precisely the function of a vaccine adjuvant. See Janeway at p.576 ("The potency of dendritic cells in activating T-cell responses provides the rationale provides the rationale for yet another strategy for

vaccinating against tumors. The use of antigen-pulsed autologous dendritic cells to stimulate therapeutically useful T-cell responses to tumors has been developed in experimental models, and there have been initial trials in humans with cancers," and Appendix A at p.7, ¶¶3-5 et seq.

11. It was my belief and opinion at the time of filing the proposal of Appendix A that elucidation of the mechanism of action of PC extract would aid in the use of pine cone extract to complement current anti-cancer therapies.

12. We demonstrated in a pilot study prior to the time of filing the proposal of Appendix A that oral delivery of pine cone extract to aged mice reduced the incidence of spontaneous tumor formation, consistent with our hypothesized mechanism of action of pine cone extract. See Appendix A at p.9, ¶5.

13. We proposed experiments in the proposal of Appendix A to test our hypothesis that exposure of murine dendritic cells to pine cone extract in vitro will prime the cells for anti-tumor activity in vivo. See Appendix A at p.5.

14. Thus, I declare that as of the date of submission of the proposal (June 1, 1999) we had conceived and were in possession of the invention as disclosed and claimed in the subject application for U.S. Patent, and were actively engaged in its reduction to practice.

15. That reduction to practice of the invention continued diligently from prior to the filing of the proposal of Appendix A to the filing of the above-captioned application for U.S. Patent, as shown by the statement of financial support provided by Ms. Diane Tippins, Business Manager of the Tampa Bay Research Institute, a copy of which is appended as Appendix C.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

Date: 7/24/03

Akiko TANAKA, Ph.D.

#7

APPENDIX A

Tampa Bay Research Institute
A NOT-FOR-PROFIT ORGANIZATION DEDICATED TO CANCER & BIOMEDICAL RESEARCH

June 1, 1999

Commander
U.S. Army Medical Research and Material Command
ATTN: MCMR-PLF (BCRP99-Announcement)
1076 Patchel Street (Building 1076)
Fort Detrick, MD 21702

Dear Commander:

Enclosed please find the original and thirty (30) copies of the proposal entitled, " Dissecting the Legendary Anti-Tumor Activity of a Pine Complex: Activating the Anti-Tumor Potential of Macrophage and Dendritic Cells."

We have used our latest non-profit indirect cost rate of 95% for this proposal. However, a new indirect cost rate will have to be established upon receiving an award.

We thank you for your consideration of our request.

Sincerely,

Diane C. Tippins

Diane C. Tippins
Business Administrator

Enclosures
Original and thirty copies of Proposal
Original and three copies of Cover Booklet
Five copies each of Technical and Public abstracts
Abstract computer disk
Nonprofit Rate agreement

P.I.
cc: William G. Bradley, Ph.D.

10900 Roosevelt Blvd., St. Petersburg, Florida 33716 • Tel. 813/576-7368 • Fax. 813/577-9862

Technical Abstract

Proposal Title: Dissecting the Legendary Anti-Tumor Activity of a Pine Complex: Activating the Anti-Tumor Potential of Macrophage and Dendritic Cells

Principle Investigator: William Guy Bradley, Ph.D.

For thousands of years humans have used the plants around them to help combat disease. The protective or healing properties of some of these plants has been associated with their ability to modulate immune function. It is therefore quite possible that many of the plants whose use has been propagated by folklore combat disease by modulating immune activity. By bolstering the immune system these products could also profoundly affect the development of human cancers. The extract from the cones of the pine, *Pinus parviflora* Sieb et. Zucc, appears to be such a product. In fact, because of its legendary anti-tumor potential, oral intake of pine cone extract is still a popular practice on the Kyushu Island of Japan. Based upon published reports and observations made recently in our laboratory, we hypothesize that the host-mediated anti-tumor activity of the pinecone extract (PC, Pine Complex) resides in its ability to alter immune function in favor of a Th1 response and to activate dendritic cells (DC) and macrophage.

To date many studies, including several of our own, have described the activity of the pinecone extract. Studies have shown that the PC is capable of inducing activation of murine and human macrophage/dendritic cells. When PBMC from a person who had taken PC or 2 weeks were stimulated with concanavalin A in vitro we observed a significant increase in cell adherence and spreading. Administration of the extract to lactating SHN mice prevented an increase of mouse mammary tumor virus (MMTV) in the milk of nursing mothers and reduced formation of MMTV-associated breast cancer. When the extract was delivered to another group of mice intraperitoneally, it was found to effectively inhibit the growth of ascites sarcoma-180 cell tumors. When the extract was incubated with the sarcoma cells in vitro, there was no detectable effect on the growth or viability of the tumor cells. From these results it was concluded that the anti-tumor activity observed in vivo reflects activation of host-mediated anti-tumor activity. Based on these results we recently investigated the ability of PC to alter immune function in aged mice. We found that treatment, by supplying PC continuously in their drinking water for 30 days, of the mice lead to alteration of splenocyte cytokine production. Specifically, PC enhanced expression of IL-12p70 by 94% and suppressed expression of IL-10 by 88%. Our findings suggest that the legendary anti-tumor might be rooted in its ability to favor a protective Th1 response, a response that would be associated with activation of macrophage and dendritic cells and would increase their ability to process and present tumor antigens.

Central to this research proposal is experiments designed to investigate the ability of PC to modulate immune function and effect the development of cancer. For these reasons we will pursue the following two aims: (1) To determine the concentrations of PC that effectively inhibit tumor development in a well established murine tumor model and (2) To determine if exposure of murine dendritic cells to PC in vitro will prime them for anti-tumor activity in vivo. Since previous investigations have demonstrated that the mere presence of macrophage and DC infiltrating into tumors correlates with a better prognosis, the ability to stimulate the production of IL-12 and to activate DC and macrophage in vivo could prove to be extremely useful in the treatment of breast cancer. To identify and characterize the cells associated with PC-induced tumor regression, we will utilize histochemical, immunohistochemical, and FACS analysis. To enhance the examination of tumor development and visualization of tumor cells and tumor margins we will use tumor cell lines that have been engineered to express high levels of green fluorescent protein (GFP).

Since the use of PC to combat cancer in humans has been predominately based upon anecdotal evidence there exists a need for well-controlled scientific experiments. By performing a thorough investigation of PC's affects on the immune system and its anti-cancer activity, it is quite possible that we may substantiate the anecdotal reports and thereby move the use of PC from the realm of folklore into the complement of therapies used to combat diseases like breast cancer.

Public Abstract

Proposal Title: Dissecting the Legendary Anti-Tumor Activity of a Pine Complex: Activating the Anti-Tumor Potential of Macrophage and Dendritic Cells
Principle Investigator: William Guy Bradley, Ph.D.

For thousands of years humans have used the plants around them to help combat disease. The protective or healing properties of some of these plants has recently been associated with their ability to modulate immune function. It is therefore quite possible that many of the plants whose use has been propagated by folklore combat disease by modulating the immune system. By bolstering the immune system these products could also profoundly affect the development of human cancers. An extract from pinecones appears to be such a product. In fact, because of its legendary anti-tumor potential, the drinking of a tea made from the pinecone is still a popular practice on the Kyushu Island of Japan.

Based upon published reports and observations made recently in our laboratory, we hypothesize that the anti-cancer activity of an extract from pine cones (PC, Pine Complex) resides in its ability to alter immune function and to specifically stimulate the activation of cells capable of recognizing and destroying cancer cells. Previous research has demonstrated that within well-controlled experiments the pinecone extract is capable of combating a number of diseases, including breast cancer. Recent research performed in our laboratory has found that the extract is able to significantly stimulate the immune system of aged mice (we chose aged mice because the incidence of cancer and other diseases in humans increases greatly as we age) to secrete factors that would be beneficial in the establishment of an anti-cancer immune response.

Central to this research proposal is experiments designed to investigate the ability of PC to stimulate immune function and suppress or prevent the development of cancer. For these reasons we will pursue the following two aims: (1) To determine the concentration of PC that effectively inhibits cancer production in a well established mouse model and (2) To determine if we can activate the anti-cancer potential of immune cells with PC in the test tube and then by injecting them directly into a tumor induce the regression of the cancer.

Since the use of PC to combat cancer in humans has been predominately based upon anecdotal evidence there exists a need for well-controlled scientific experiments. By performing a thorough investigation of PC's affects on the immune system and its anti-cancer activity, it is quite possible that we may substantiate the anecdotal reports and thereby move the use of PC from the realm of folklore into the complement of therapies used to combat diseases like breast cancer.

Statement of Work

Bradley, William G.

Dissecting the Legendary Anti-Tumor Activity of a Pine Complex: Activating the Anti-Tumor Potential of Macrophage and Dendritic Cells

Task 1: To determine the concentrations of PC that effectively inhibit tumor development in BALB/c murine tumor model (months 1-24)

- Create the C51 tumor cell line that constitutively express the green fluorescent protein at high levels (months 1-7)
- Establish minimal number ofCT-26 and C51 cells required to induce tumors in 100% of the mice within 2 weeks (months 7-9)
- Begin testing different concentrations of PC for its ability to inhibit or reduce tumor formation (months 9-15)
- Examine tumor architecture by using histological and immunohistological techniques, and FACS analysis (months 9-21)
- Statistical analysis of results (months 21-24)

Task 2: To determine if exposure of murine dendritic cells to PC in vitro will prime them for anti-tumor activity in vivo (months 24-60).

- Isolation and characterization of dendritic cells (months 24-30)
- Determination of appropriate concentration of PC to activate the isolated dendritic cells in vitro (months 24-30)
- Establish appropriate concentration of PC that inhibits tumor formation in <50% of the mice (months 30-33)
- Begin testing for the most effective timing of injection of the in vitro activated dendritic cells (months 33-45)
- Examine tumor architecture by using histological and immunohistological techniques, and FACS analysis (months 45-57)
- Statistical analysis of results (months 57-60)

Proposal Relevance and Impact Statement

An extract from the cones of the pine, *Pinus parviflora* Sieb et. Zucc, has been used over the centuries by people in China, Germany, Greece, and Japan to combat diseases. Its recent rediscovery was based upon its association with the significant reduction in the incidence of cancer in people that used it as a component of their traditional medicine. Since the use of the extract to combat cancer in humans has been predominately based upon anecdotal evidence there exists a need for well-controlled scientific experiments. By performing a thorough investigation of PC's affects on the immune system and its anti-cancer activity, it is quite possible that we may substantiate the anecdotal reports and thereby move the use of PC from the realm of folklore into the complement of therapies used to combat diseases like breast cancer.

Proposal Body

I. Background

Breast cancer is the second leading cause of deaths in women. It is estimated that I in 11 women will eventually develop this disease. While science has made significant progress in the early detection and treatment of breast cancer, many of the currently approved treatments negatively impact the immune system (1-5). Unfortunately, impaired immune function is associated with poor prognosis in breast cancer (6-8). It would therefore seem logical that bolstering the immune system and specifically inducing an anti-cancer immune response would improve the outcome of cancer therapy. This is especially true in the aged population, where there is recognized a strong correlation between the increase incidence of cancers and a senescing immune system.

Immunosenescence

It has been well established that as humans and mice age, overall immune function declines. Concomitant with this decline is an increase in the incidence of cancer and other diseases. It has therefore been speculated that prevention or reversal of this decline in immune function could possibly reduce the incidence of cancers and other age-related diseases (9-12). One of the immunologic changes associated with aging is T cell dysfunction. This T cell dysfunction is recognized as the reduced ability of aged T cells to proliferate in response to mitogens and antigens and as an imbalance in the T helper (Th) patterns of cytokine expression (13). This aging-associated T cell dysfunction is associated with thymus involution and an increase in the CD4+ T cell population having a memory phenotype (14). When the peripheral blood mononuclear cells (PBMC) from young and adult humans and PBMC and splenocytes from young and adult mice are stimulated in vitro, they produce a T helper type I (Th1) pattern of cytokine expression. However, when the PBMCs from aged humans and PBMCs and splenocytes from aged mice are stimulated in vitro, they produce a T helper type 2 (Th2) pattern of cytokine expression (15-20). The Th1 pattern of cytokine expression is characterized by the production of interleukin 2 (IL-2), IL-12, and interferon gamma (IFN-γ) and is necessary for a proper cellular immune response and the ability to combat cancers, while the Th2 pattern of cytokine expression is characterized by the production ofIL-4, IL-5, IL-6, and IL-10 and is necessary for a proper humoral immune response.

Interleukin 12, Dendritic Cells and Macrophage

The expression of IL-12 is associated with the Th1 type of immune response. Interleukin 12 has been found to affect T cell and natural killer cell functions, including induction of IFN-y production and the enhancement of cell-mediated cytotoxicity, and to play a major role in the induction of the Th1 response and suppression of the Th2 response (21-23). Since IL-12 can help activate a Th1 response, it has been speculated that it could be used to treat diseases like cancer (24). Several studies in fact have demonstrated that systemic administration of IL-12 has anti-tumor effects in animal models (25-27).

It is currently believed that IL-12 induces an anti-tumor state by activating professional antigen-presenting cells (APC, macrophage and dendritic cells). In fact, it has been demonstrated that tumor antigens are presented to T cells not by the tumor cells themselves but by host APC (28). During antigen-induced immune responses, dendritic cells (DC) take up antigen, migrate through the lymphatic system or blood stream to the lymphoid organs, and present the antigen to T cells. It appears that the capacity to internalize and process antigen is a constitutive property of DC present in non-lymphoid organs (29).

In the absence of IL-12, antigen-presenting cells infiltrating a tumor can capture tumor antigens but appear to present the antigens as new epitopes arising from normal self-antigens rather than from a threatening tumor (30). In the presence of IL-12, antigenic determinants from the tumors are recovered by professional antigen presenting cells (APC) and then processed and delivered to cytotoxic T cells. It appears that DC and macrophage are the only cells that can do this in response to IL-12 (31,32). This process of antigen processing and

presentation is so critical to establishing an anti-tumor response that the mere presence of macrophage and DC infiltrating tumors has been correlated with a better prognosis (33). Therefore, the ability to stimulate the production of IL-12 and to activate DC and macrophage could prove to be extremely useful in the treatment of breast cancer. In several studies it has been demonstrated that activation of DC in vitro, in the presence of tumor antigen and specific cytokines, can induce tumor-specific cytotoxicity when used as a tumor vaccine (34-37). But how do we accomplish this in vivo? Based upon years of accumulating anecdotal evidence and preliminary studies in our laboratory, we believe that an extract from the cones of pine may be able to inhibit the production of IL-10, stimulate the production of IL-12, and activate DC and macrophage in vivo, thereby inducing anti-tumor activity.

Origin of the Pinecone Extract

For thousands of years humans have used the plants around them to help combat disease (38). The protective or healing properties of some of these plants has recently been associated with their ability to modulate immune function. It is therefore quite possible that many of the plants or plant products whose use has been propagated by folklore, combat disease by modulating the immune system. By bolstering the immune system these products could also profoundly affect the development of human cancers. The extract from the cones of the pine, *P. parviflora* Sieb et. Zucc, which contains pheylpropenoid polymers complexed with polysaccharides, appears to be such a product. In fact, because of its legendary anti-tumor potential, oral intake of the *Pinus parviflora* Sieb et. Zucc extract is still a popular practice on the Kyushu Island of Japan (39).

In vitro Activity of the *Pinus parviflora* Sieb et. Zucc

Extract To date several studies, including several of our own, have described the in vitro activity of the *Pinus parviflora* Sieb et. Zucc extract. Studies have shown that an alkaline extract from *Pinus parviflora* Sieb et. Zucc is capable of inducing the activation of a mouse macrophage cell line (40). The extract was found to induce morphological spreading and stimulated the NBT-reducing activity of peritoneal macrophage. When human peripheral blood monocytes were primed with the extract and then stimulated with LPS, a significant increase in TNF mRNA expression was observed (41). Recently, when we stimulated human PBMC in vitro with increasing concentrations of either concanavalin A or a commercially available pinecone extract, (PC; Pinextra, Vitamune, Tampa, Florida), we detected a very highly significant increase in the attachment and spreading ofdendritic/macrophage in the samples treated with PC (manuscript in preparation).

The extract from *Pinus parviflora* Sieb et Zucc has also been shown to inhibit virion-associated RNA dependent RNA polymerase, inhibit both forward- and reverse-transcription of HIV in vitro, inhibit HIV replication in T cell and monocytic cell lines in vitro (42), and inhibit plaque-formation in cells infected with either influenza or herpes simplex virus (43,44). While these in vitro activities are quite interesting, we are presently focusing our activities toward understanding how the extract works in vivo.

In vivo Activity of the Pinus parviflora Sieb et. Zucc Extract

To date, only a few studies describing the in vivo activity of the pinecone extract have been reported. These studies have reported the antimicrobial, antiparasitic, antiviral, and antitumor activity of an alkaline extract from the *Pinus parviflora* Sieb et. Zucc. Oh-Hara et al. demonstrated that pretreatment of mice with Fraction VI (Fr. VI) of the *Pinus parviflora* Sieb et. Zucc extract was capable of inducing a potent antimicrobial response against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Candida albicans* (45,46). Abe et al. reported that similar fractions of the pinecone extract were capable of protecting both young and infant mice from parasitic (*Hymenolepis nana*) infection (47).

Analysis of Toxicity

In 1987, Sakagami et al. described the preparation of several biologically active fractions from *Pinus parviflora* Sieb et Zucc (48). When acute and subacute toxicity tests were performed on Fr VI, it was discovered to be

moderately toxic when delivered intravenously to mice. The LD5o value of the extract on these mice was approximately 60 mg/kg. However, when Fr VI was delivered orally to mice, no toxic effects were noted at doses as high as 405 mg/kg (41). When doses ranging from 5 mg/kg to 405 mg/kg was administered daily for 30 days there was observed no alteration of the hepatic P-450 system (41).

Anti-tumor activity of the *Pinus parviflora* Sieb et Zucc Extract

The administration of Fr. VI of the extract, either intravenously or orally to lactating SHN mice (a strain of mice that produces mouse mammary tumor virus in their milk and develops MMTV-associated breast cancer), appeared to prevent an increase of mouse mammary tumor virus (MMTV) in the milk of nursing mothers (49). When mice that had spontaneously developed mammary tumors were treated by intravenous injection of Fr. VI, it was found that the treatment effectively reduced the activity of thymidylate synthetase and thymidine kinase in cells derived from the tumors (50).

When the *Pinus parviflora* Sieb et Zucc extract was delivered intraperitoneally at a concentration of 20 mg/ kg it was found to effectively inhibit the growth of ascites sarcoma-180 cell tumors in mice and increased the mean survival time from 16-18 days (control group) to 31-51 days (treated group) (48). When the *Pinus parviflora* Sieb et Zucc extract (at concentrations up to 500 ug/ml) were incubated with the sarcoma-180 cells in vitro, there was no detectable effect on the growth or viability of the tumor cells. From these results it was concluded that since the extract had no apparent effect on the tumor cells in vitro, the anti-tumor activity observed in vivo reflects activation of host-mediated anti-tumor activity (48).

Because the report by Sakagami et al. suggested that the extract's anti-tumor activity is likely to be associate with the ability to modulate immune function, we recently investigated the potential of oral administration of PC to alter immune function. Since we realized that enhancement of immune function and the induction of anti-tumor activity in the aged would be especially important, we investigated the ability of oral administration of PC to aged mice to alter the ability of their splenocytes to produce a number of cytokines (manuscript in preparation). We found that oral administration of PC to aged B6C3F1/Nnia mice profoundly altered cytokine production by their concanavalin A-stimulated splenocytes. Treatment, by supplying PC continuously in their drinking water for 30 days, of the aged mice lead to enhanced expression ofIL-12p70 (94% above levels in non-treated mice) and suppression of IL-10 (88% of the levels produced by non-treated mice). Our findings that the oral administration of PC to aged mice suppressed the production of IL-10 and enhanced the production of IL-12, suggest that the legendary anti-tumor potential ascribed to the pine cone extract might be rooted in its ability to counteract the Th2 pattern of cytokine expression and favor a protective Th1 response. Such a response, if it occurs in vivo, would likely favor activation of macrophage and dendritic cells and increase their ability to process and present tumor antigens.

In a small pilot study (only 5 mice per treatment group) currently in progress, the oral delivery of PC to aged B6C3F1/Nnia mice (23-25 months old) appears to have enhanced their longevity and overall health. While several mice in the group receiving only water have developed spontaneous tumors and expired, none of the mice receiving PC have developed spontaneous tumors nor have any expired. The mice are currently 30-32 months old (expected life span for B6C3F1/Nnia is reported to be 35-36 months). While these findings are very interesting, this study utilized a minimum of mice and was designed to look only at longevity and not specific alterations of immune function or the establishment of an anti-cancer response.

While the anti-tumor activity of PC in humans is legendary on the Kyushu Island of Japan, and while the health benefits of PC that have been described in the Greek Herbal of Dioscorides (published in the first century A.D.) are compelling, much of the evidence remains anecdotal. By performing a thorough investigation of PC's affects on the immune system and its anti-cancer activity, it is quite possible that we may end up substantiating the anecdotal reports and thereby move the use of PC from the realm of folklore into the complement of therapies

used to combat cancer.

II. Hypothesis/Rationale/Purpose

Based upon published reports and observations recently made in our laboratory, we hypothesize that the host mediated anti-tumor activity of PC resides in its ability to activate dendritic cells and macrophage. Since the rediscovery of the pinecone extract was due to its legendary anti-tumor activity we anticipate that by elucidating its mechanism of action the use of PC to complement current anti-cancer therapies is forthcoming.

III. Objectives (specific aims)

Central to this research proposal is experiments designed to investigate the ability of PC to modulate immune function and effect the development of cancer. For these reasons we wish to pursue the following aims.

Aim 1. To determine the concentrations of PC that effectively inhibits tumor development in the BALB/c murine tumor model. The rediscovery of the *Pinus parviflora* Sieb et. Zucc extract was due to its remarkable anti-cancer activity in humans. In order to dissect how PC effects tumor development, we will determine the concentrations of PC that effectively suppress tumor development in the widely accepted murine tumor model (BALB/c CT26 adenocarcinoma). We will utilize histochemical, immunohistochemical, and FACS analysis to characterize and identify the cells involved in tumor suppression/regression. To enhance the histological examination of tumor development we will use tumor cell lines that have been engineered to express high levels of green fluorescent protein (GFP). The constitutive expression of GFP in the tumor cells will allow direct visualization of tumor cells and tumor margins under fluorescent microscopy.

Aim 2. To determine if exposure of murine dendritic cells to PC in vitro will prime them for anti-tumor activity in vivo. Since it has been established that in vitro activation of tumor antigen-pulsed DC are capable of inducing tumor-specific cytotoxicity and providing protection against subsequent inoculation of tumor cells in vivo, we will determine if exposure of dendritic cells to PC in vitro, followed by continuous oral administration of PC, will prime them for establishing an anti-tumor state in vivo. We will utilize histochemical, immunohistochemical, and FACS analysis to characterize and identify the cells involved in tumor suppression/regression. To enhance the histological examination of tumor development we will use tumor cell lines that have been engineered to express high levels of green fluorescent protein (GFP). The constitutive expression of GFP in the tumor cells will allow direct visualization of tumor cells and tumor margins under fluorescent microscopy.

IV. Methods

Aim 1. To determine the concentrations of PC that effectively inhibits tumor development in BALB/c murine tumor model.

Rationale: Since the remarkable anti-cancer activity of PC has been predominately based upon anecdotal evidence there exists a need for well controlled scientific experiments to substantiate its legendary anti-cancer activity. In order to mimic the development of cancer in humans in a more controlled environment, we will use the well-characterized BALB/c CT26 and C51 (adenocarcinomas) tumor model to investigate the concentrations of PC that effectively delays, suppresses, or induces regression of tumor development. The results obtained from this series of experiments will allow us to better formulate the complementary use of PC in the treatment of cancers.

Experimental Design: BALB/c mice will be obtained from Jackson Laboratories. Mice will be housed in

AAALAC approved facilities associated with the University of South Florida (Tampa, Florida). This research protocol has been approved by the USF's IACUC and has been assigned the file # 1442. Mice will be grouped and treated with PC at the concentrations shown in Table I by supplying it continuously in their drinking water. Tumor formation will be initiated by injecting 10^4 CT-26 or C51 cells subcutaneous in their flank. This dose of tumor cells reproducibly induces detectable tumor formation in 80-100% of mice within 10 days of injection. Treatment with PC will begin either 2 weeks prior to, at the same time as, or I week after injection with 10^4tumor cells. The treatment schedule will continue until tumors in the control group (0 ug/ml) expand to >10mm in diameter, after which the tumors will be removed and examined histologically using conventional and immunohistochemical stains. Specifically we will prepare frozen and paraffin sections from tissues in the vicinity of the tumor cell injection and/or resulting tumors and examine them by immunohistochemistry for infiltrating T-helper cells (CD4+/L3T4+), cytotoxic T cells (CD8+/Lyt-2+), NK cells (asialo-GMI+), B cells (CD45R+/B220+), or dendritic/macrophage/monocytes (CD86+/B7-2+). We will also look for the presence of newly activated T cells and NK cells displaying the very early activation antigen, CD69. The frozen and paraffin sections and the hematoxylin and eosin staining of paraffin sections will be performed by staff of All Children's Hospital's Department of Pathology (St. Petersburg, FL). For histologic analysis, tissues will be fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 2-5um, and stained with hematoxylin-eosin. For immunocytochemistry, tissues will be embedded in OCT compound, snap-frozen in liquid nitrogen, and preserved at -80°C until sectioned. Cryostat sections of 5 um will be fixed in acetone and immunostained with purified rat mAb to: CD4/L3T4, CD8/Lyt-2, asialo-GMI, CD45R/B220, CD86/B7-2 or CD69 (Southern Biotechnology Associates, Inc). Each of these antigen specific mAbs will be conjugated with phycoerythrin (R-PE). To develop the tissue sections, reagents available in the Vectastain ABC kits (Vector Laboratories) will be used. The tissue sections will be counterstained with hematoxylin and then examined by light and fluorescence microscopy using an Nikon Eclipse E600 microscope equipped with a Microflex U-III camera system.

To enhance the histological examination of tumor development we will use CT26 and C51 tumor cells that have been engineered to express high levels of green fluorescent protein, thereby allowing for direct visualization of tumor cells under fluorescent microscopy. Using fluorescent microscopy we will be able to detect the tumor and its margins and we might also be able to detect small numbers of tumor cells at the site of injection in the mice that appear to have suppressed tumor formation. When we superimpose the results from light microscopy with the fluorescent microscopy it will be easy to determine the specific locations of tumor infiltrating cells.

To determine how PC may have influenced tumor production we will also analyze cytokine expression by the splenocytes isolated from each mouse that either: (1) suppressed tumor formation, (2) induced tumor stasis, or (3) induced tumor regression. We will compare the levels of cytokine production in these 3 groups of mice to the levels detected in the control mice with tumors.

To examine the effects of oral administration of PC on cytokine expression we will analyze the ability of splenocytes and lymph node cells to respond to anti-CD3 mAb, concanavalin A (ConA), or lipopolysaccharide (LPS) mitogen in vitro. Mice will be sacrificed, spleens and lymph nodes will be removed aseptically, and single cell suspensions will be prepared individually for each mouse using a Stomacher 80 Laboratory Blender (Seward Medical, London, England). Cell suspensions will be passed through a fine mesh filter prior to use. The cells will be enumerated using a Coulter Counter and viability will be assessed by examination of trypan blue dye exclusion using a hemocytometer.

Spenocytes and lymph node cells will be diluted to 2 x 10^6 per ml in serum free media (media is RPMI 1640 containing, lx Nutridoma SR (Boehringer Mannheim), 10 mM Hepes, 1% L-glutamine, 1% penicillin/streptomycin, and 50 uM 2-mercaptoethanol) containing either 10ug/ml anti-CDS mAb (clone 145-2CII), 5ug/ml ConA or 20ug/ml LPS and then incubated at 37C in an atmosphere containing 5% C02.

Triplicate cell cultures will be prepared from each cell source. After 48 hr the supernatants will be harvested and stored at -80°C. The amount of IL-1, IL-2, IL-3, IL-4, IL-6, IL-10, IL-12p70, TNF-α, and IFN-γ in the supernatants will be measured by ELISA using commercially available kits. The assays will be performed according to the manufacturer's instructions.

All in vitro cytokine expression experiments will be performed independently in triplicate. The significance of differences between the levels of cytokine expression in each treatment group will be statistically evaluated using a two-tailed Student's t-test. The tumor takes will be analyzed by Fisher's exact method and the latency/survival time will be analyzed using the two-tailed Wilcoxon's signed rank test.

Expectations and Feasibility: The use of CT26 and C51 tumor cell lines in BALB/c as tumor models have been well established and are extremely reproducible. The CT26 and C51 are colon adenocarcinoma cell lines derived from BALB/c mice treated with N-nitroso-N-methylurethane and 1,2-dimethylhydrazine, respectively (51). We choose to use these tumor cell lines because they were both derived from BALB/c mice and they share transplantation and CTL-recognition antigens (52). However, CT26 is significantly more resistant than C51 to systemic administration of IL-12 (53), an experimental treatment that has been demonstrated to improve the survival of mice bearing a variety of tumors (54). The greater resistance of CT26 to cytotoxic attack is likely to be associated with the ability of CT26 to induce IL-10 production by the infiltrating T cells when IL-4 is present (32). Since our previous investigations demonstrate that the major effect of PC is to induce IL-I 2 production and suppress IL-10 production in vitro in stimulated splenocytes, it is likely that PC will also stimulate the production of IL-12 and suppress the production of IL-10 in vivo and lead to tumor regression. Having many years of experience working with such tumor models we do not anticipate any technical difficulties. The CT26/GFP cell line will be provided by Dr. Robert Siliciano (John's Hopkins University). According to the observations in Dr. Siliciano's laboratory, the presence of GFP in the CT26 cells does not appear to significantly affect their ability to develop tumors in vivo or the immune response against such tumors.

The results obtained from this series of experiments may provide scientific evidence that supports the historical claims that PC has or induces anti-tumor activity or at least will provide a foundation from which we can begin investigating PC's specific mechanism of action.

Aim 2. To determine if exposure of murine dendritic cells to PC in vitro will prime them for anti-tumor activity in vivo.

Rationale: Previous investigations have demonstrated that the mere presence of macrophage and DC infiltrating into tumors correlates with a better prognosis (33). Therefore, the ability to stimulate the production of IL-I 2 and to activate DC and macrophage in vivo could prove to be extremely useful in the treatment of cancer. In several studies it has been demonstrated that activation of DC in vitro, in the presence of tumor antigen and specific cytokines, can induce tumor-specific cytotoxicity when used as a tumor vaccine (34-37). In Aim I we will determine if PC's anti-tumor activity is associated with its ability to induce macrophage and DC infiltration into tumors. Even if we find that oral administration of PC is capable of 100% tumor suppression/regression we are aware that it may not work for all types of cancer and that an alternative protocol should be investigated.

In Aim 2 we would like to determine the feasibility of combining the oral administration of PC (Aim 1) with in vitro dendritic cell activation in an attempt to maximize the stimulation of anti-tumor activity. However, in contrast to previous investigations where the DC were "loaded" in vitro with mock tumor antigens, we wish to determine if dendritic cells activated in vitro by PC and then injected directly into the tumor or at a distant site, can recognize the tumor, process the tumor antigens, and establish anti-tumor activity. This scenario may be more closely representative of the human condition and therefore needs to be investigated.

Experimental Design

For these experiments we will utilize 2 groups of BALB/c mice (8 weeks old). One group will be the source of splenic dendritic cells and the other will be the tumor test group.

Activation of DC in vitro. Mice will be sacrificed, spleens will be removed aseptically, and single cell suspensions will be prepared using a Stomacher 80 Laboratory Blender (Seward Medical, London, England). Cell suspensions will be passed through a fine mesh filter prior to use. Low-density cells will be isolated by centrifugation over a 60% Percoll gradient (density = 1.076). Dendritic cells will be further enriched by differential adherence by incubating the cells on 100mm tissue culture plates in medium containing 5% fetal calf serum (FCS) for 2 hours at 37°C. The nonadherent cells will be removed and the remaining cells will be cultured overnight in medium containing 5% FCS. Twenty-four hours later, floating cells (DC) will be collected. The cells will be transferred to media containing 5% FCS and PC at 50ug/ml. The cells will be cultured for 24-48 hours. The cells will be harvested by scraping them from the tissue culture dish and then washed with 0.9% saline (normal saline). The cells will be suspended in normal saline at a concentration of I x 10" cells/ml.

Oral administration of PC. The dose of PC that was found in Aim I to inhibit tumor formation by <50% when delivery was initiated at the same time as tumor cell injection will be used.

Tumor Induction/Treatment. On Day 0 fifty mice will be injected with 10^4 CT26/GFP cells subcutaneous in the flank and then separated into 5 treatment groups (Figure 1). On Day 0, Group I will receive an intratumor injection of 10^5 PC-treated dendritic cells and PC in their drinking water. Two days post tumor cell injection, Group 2 will receive an intratumor injection of 10^5 PC-treated dendritic cells and PC in their drinking water. Four days post tumor cell injection, Group 3 will receive an intratumor injection of 10^5 PC-treated dendritic cells and PC in their drinking water. Six days post tumor cell injection, Group 4 will receive an intratumor injection of 10^5 PC-treated dendritic cells and PC in their drinking water. Eight days post tumor cell injection, Group 5 will receive an intratumor injection of 10^5 PC-treated dendritic cells and PC in their drinking water. The mice will be analyzed on a daily basis for tumor growth. The size of the tumors will be determined with small calipers by measuring the diameter. The experiment will continue until the tumors are 20 mm in diameter or if no tumors have developed after 60 days.

Statistical analysis. The tumor takes will be analyzed by Fisher's exact method and the latency/survival time will be analyzed using the two-tailed Wilcoxon's signed rank test.

Expectations and Feasibility: The experiments described in Aim 2 will allow us to determine if the combination of orally administered PC with the intratumor injection of in vitro PC-stimulated dendritic cells is effective at inducing anti-tumor activity. The experimental design will allow us to test a variety of scenarios. By injecting the DC and beginning oral administration of PC 0 and 2 days post tumor cell injection we will mimic a condition similar to that in patients with a minimal tumor burden (either because of early detection or resection of the majority of the tumor by surgery). By injecting the DC and beginning oral administration of PC 4, 6, and 8 days post tumor cell injection we will mimic a condition similar to that found in patients where significant tumor growth has occurred.

If we fail in Aim 1 to find an effective concentration of PC that induces tumor suppression or regression we will then determine if the combination treatment (Aim 2) using PC is effective at inducing anti-tumor activity. However, since the rediscovery of the pinecone extract was due to its legendary anti-tumor activity we anticipate that positive results will be obtained from Aim 1. The results obtained from both Aim 1 and Aim 2 will provide us information that will help in deciphering the mechanisms by which PC induces anti-tumor activity.

V. Figures and Tables

Table 1. Treatment schedule for each group of mice injected with 10^4 tumor cells

Timing of PC Treatment	Number of mice to be utilized in each group				
	Group 1	Group 2	Group 3	Group 4	Group 5
2 weeks prior to tumor cell injection	10	10	10	10	10
Same time as tumor cell injection	10	10	10	10	10
1 week post tumor cell injection	10	10	10	10	10

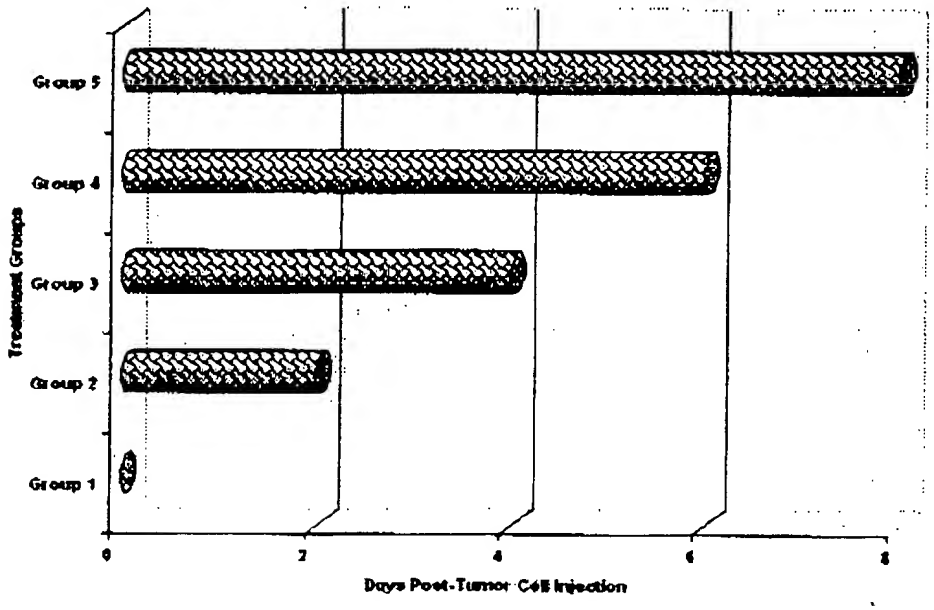

Figure 1. Mice in each treatment group will be injected with tumor cells on Day 0 and then 2, 4, 6, or 8 days later they will receive an injection of PC-activated dendritic cells and will start receiving PC continuously in their drinking water.

References

1. Hengst JCD, Mitchell MS. Principle of combining biological response modifiers with cancer chemotherapy. In: Hellmann K, Carter SK (eds) Fundamentals of cancer chemotherapy. McGraw Hill, New York, pp 64-76. 1980.
2. Park SK, Brody JI, Wallace HA, Blakemore WS. Immunosuppressive effect of surgery. Lancet 1:53.1971.
3. Stjemsward J (1974) Decreased survival related to irradiation postoperatively in early operable breast cancer. Lancet 11:1285.
4. Toivanen A, Nordman E. Long-term effect of postoperative irradiation of the immune functions in patients with mammary carcinoma. Acta Radiol Oncol 20:119,1981.
5. Verdecchia MC, Prete SP, Marcotullio D, Bonmassar E, De Vecchis L. NK cell activity after surgery in head-neck cancer patients. J Exp Clin Cancer Res 12:141, 1993.
6. Alder A, Stein JA, Ben-Efraim S. Immunocompetence, immunosuppression, and human breast cancer I and III. Cancer 45:2061,1980.
7. Bolton PM, Teasdale C, Mander AM, James SL, Davidson JM, Whitehead RH, Newcombe RG, Hughes LE. Immune competence in breast cancer: relationship of pretreatment immunologic tests to diagnosis and tumor stage. Cancer Immunol Immunother 1:251, 1976.
8. Eilber FR, Morton DI. Impaired immunological reactivity and recurrence following cancer surgery. Cancer 25:362,1970.
9. Song L., Kim YH, Chopra RK, Proust JJ, Nagel JE, Nordin AA, Alder WH. Age-related effects in T cell activation and proliferation. Exp. Gerontol. 28:33-321,1993.
10. Murasko DM, Weiner P, Kaye, D. Decline in mitogen induced proliferation of lymphocytes with increasing age. Clin. Exp. Immunol. 70:440-448, 1987.
11. Makinodan T, Kay MMB. 1980. Age influence on the immune system. Adv. Immunol. 29:287, 1980.
12. Hicks MJ, Jones J, Thies AC, Weigle K, Minnick J. Age related changes in mitogen-induced lymphocyte function from birth to old age. Amer. J. Clin. Path. 80:159-163,1983.
13. Castle S, Uyemura K, Wong W, Modlin R, Effros R. Evidence of enhanced type 2 immune response and impaired upregulation of a type I response in frail elderly nursing home residents. Mech Ageing Dev. 94:7-16,1997.
14. Linton PJ, Haynes I, Klinman NR, and Swain SL. Antigen -independent changes in naive CD4 T cells with aging. J. Exp. Med. 184:1891-1900, 1996.
15. Hobbs MV, Ernst DN. T cell differentiation and cytokine expression in late life. Dev Comp Immunol. 21:461-470,1997.
16. Frasca D, Doria G. Recombinant cytokines as an approach to immune reconstitution in aging. Dev Comp Immunol 21:525-530, 1997.
17. Mysliwska J, Bryl E, Foerster J, Mysliwski A. Increase of interleukin 6 and decrease of interleukin 2 production during the aging process are influenced by the health status. Mech Ageing Dev. 100:313-328, 1998.
18. O'Mahony L, Holland J, Jackson J, Feighery C, Hennessy TP, Mealy K. Quantitative intracellular cytokine measurement: age-related changes in proinflammatory cytokine production. Clin Exp Immunol. 113:2133219,1998.
19. Rink L, Cakman I, Kircher H. Altered cytokine production in the elderly. Mech Ageing Dev. 102:199209,1998.
20. Kurashima C, Utsuyama M. Age-related changes of cytokine production by murine helper T cell subpopulations. Pathobiology 65:155-162.1997.
21. Seder, RA and Paul, WE. Acquisition of lymphokine-producing phenotype by CD4+ T cells. Annu. Rev. Immunol. 12:635-674,1994.
22. Lee SM, Suen Y, Qian J, Knoppel E, Cairo MS. The regulation and biological activity of interleukin 12. Leuk Lymphoma 29:427-438,1998.

23. Manetti R, Parronchi P, Giudizi MG, Piccini MP, Maggi, E, Trinchieri G, and Romagnani S. Natural killer cell stimulatory factor (IL-12) induces TH I-specific immune responses and inhibits the development ofIL4 producing TH cells. J. Exp. Med 177:1199-1204, 1993.
24. Gately MK, Renzetti LM, Magram J, Stem AS, Adorini L, Gubler U, Presky DH. The interleukm-12/interleukin12-receptor system: role in normal and pathologic immune responses. Annu Rev Immunol 16:495-521, 1998.
25. Brunda MS, Luistro L, Warrier RR, Wright RB, Hubbard B, Murphy M, Wolf SF, and Gately MF. Antitumor and antimetastatic activity of interleukin 12 against murine tumors. J Exp Med 178:1223, 1993.
26. Lotze MT. Recombinant IL-12 administration induces tumor regression in association with IFN-y production. J Immunol 153:1697, 1994.
27. Voest EE, Kenyon BM, O'Reilly MS, Truitt G, D'Amato RJ, Folkman J. Inhibition of angiogenesis in vivo by interleukin 12. J Natl Cancer Inst 87:581, 1995.
28. Huang AYC, Golumbek P, Ahmadzadeh M, Jaffee E, Pardoll D, Levitsky H. Role of bone marrow derived cells in presenting MHC class I-restricted tumor antigens. Science 264:961, 1994.
29. Romani N, Koide S, Crowley M, Witmer-Pack M, Livingstone A, Fayhman CG, Inaba K, Steinman RM. Presentation of exogenous protein antigen by dendritic cells to T cell clones. Intact protein is presented best by immature, epidermal Langerhans cells. J Exp Med 169:1169, 1989.
30. Inaba K, Pack M, Inaba M, Sakuta H, Isdell F, Steinman RM. High levels of major histocompatibility complex II-self peptides on dendritic cells from the T cell areas of lymph nodes. J Exp Med 186:665-672, 1997.
31. Norbury CC, Hewlett LJ, Prescott AR, Shastri N, Watts C. Class I MHC presentation of exogenous soluble antigen via macropinocytosis in bone marrow macrophages. Immunity 6:783-791, 1995.
32. Paglia P, Stoppacciaro A, Parmiani G, Colombo MP. Genetic modification of murine carcinoma influences the recruitment and T cell costimulatory properties of dendritic cells at tumor site. J Invest Dermatol 109:261,1995.
33. Becker Y. Anticancer role of dendritic cells in human and experimental cancers - a review. Anticancer Res 12:511-520,1992.
34. Flamand V, Sornasse T, Thielemans K, Demanet C, Bakkus M, Bazin H, Tielemans F, Leo 0, Urbain J, Moser M. Murine dendritic cells pulsed in vitro with tumor antigen induce tumor resistance in vivo. Eur J Immunol 24:605,1994.
35. Mayordomo JI, Zorina T, Storkus WJ, Zitvogel L, Celluzzi C, Falo LD, Melief CJ, Ildstad ST, Kast WM, Deleo AB, Lotze MT. Bone marrow-derived dendritic cells pulsed with synthetic tumour peptides elicit protective and therapeutic antitumor immunity. Nat Med 1:1297, 1995.
36. Paglia P, Chiodoni C, Rodolfo M, Colombo MP. Murine dendritic cells loaded in vitro with soluble protein prime cytotoxic T lymphocytes against tumor antigen in vivo. J Exp Med 183:317, 1996.
37. Porgador A, Snyder Gilboa E. Induction of antitumor immunity using bone marrow-generated dendritic cells. J Immunol 156:2918, 1996.
38. A. Sofowora, Medicinal Plants and Traditional Medicine in Africa. Wiley, New York, p.9, 1992.
39. Lai PK, Donovan J, Takayama H, Sakagami H, Tanaka A, Konno K, Nonoyama M. Modification of human immunodeficiency viral replication by pine cone extracts. AIDS Research and Human Retroviruses 6:205-217,1990.
40. Kikuchi K, Sakagami H, Fujinaga S, Kawazoe Y, Oh-Hara T, Ichikawa S, Kurakata Y, Takeda M, Sato T. Stimulation of mouse peritoneal macrophages by lignin-related substances. Anticancer Res 11:841-845, 1991.
41. Sakagami H, Kawazoe Y, Komatsu N, Simpson A, Nonoyama M, Konno K, Yoshida T, Kuroiwa Y, Tanuma S. Antitumor, antiviral and immunopotentiating activities of pine cone extracts: potential medicinal efficacy of natural and synthetic lignin-related materials (review). Anticancer Res. 11:881-888, 1991.
42. Tamura Y, Lai PK, Bradley WG, Konno K, Tanaka A, Nonoyama M. A soluble factor induced by an extract from *Pinus parviflora* Sieb et Zucc can inhibit the replication of human immunodeficiency virus in vitro.

Proc Natl Acad Sci U S A 88:2249-2253, 1991.
43. Nagata K, Sakagami H, Harada H, Nonoyama M, Ishihama A, Konno K. Inhibition of influenza virus infection by pine cone antitumor substances. Antiviral Research 13:11-21, 1990.
44. Fukuchi K, Sakagami H, Ikeda M, Kawazoe Y, Oh-Hara T, Konno K, Ichikawa S, Hata, N, Knodo H, Nonoyama M. Inhibition of herpes simplex virus infection by pine cone antitumor substances. Anticancer Res. 9:313-317,1989.
45. Harada H, Sakagami H, Konno K, Sato T, Osawa N, Fujimaki M, Komatsu N. Induction of antimicrobial activity by antitumor substances from pine cone extract of Pinus parvifloria Sieb. et Zucc. Anticancer Research 8:581-588,1988.
46. Oh-Hara T, Sakagami H, Kawazoe Y, Kaiya T, Komatsu N, Ohsawa N, Fujimaki M, Tanuma S, Konno K. antimicrobial spectrum of lignin-related pine cone extracts of *Pinus parviflora* Sieb. et Zucc. In Vivo. 4:7-12,1990.
47. Abe M, Okamoto K, Konno K, Sakagami H. Induction of antiparasite activity by pine cone lignin-related substances. In Vivo 3:359-362, 1989.
48. Sakagami H, Ikeda M, Unten S, Takeda K, Murayama J-I, Hamada A, Kimura K, Komatsu N, Konno K. Antitumor activity of polysaccaride fractions from pine cone extract of Pinus parviflora Sieb. et Zucc. Anticancer Research 7:1153-1160,1987.
49. Nagasawa H, Iwai Y, Iwai M, Suzuki A, Imai S. Suppression by a pine cone extract of Pinus parviflora Sieb et Zucc of mammary tumour virus in milk of mice. Anticancer Res 12:845-847, 1992.
50. Nagasawa H, Sakamoto S, Sawaki K. Inhibitory effect of lignin-related pine cone extract on cell proliferating enzyme activity of spontaneous mammary tumours in mice. Anticancer Res 12:501-503, 1992.
51. Corbett TH, Griswold DP Jr., Roberts BJ, Peckham JC, and Schabel FM Jr. Tumor induction relationships in development of transplantable cancers of the colon in mice for chemotherapy assay, with a note on carcinogen structure. Cancer Res. 35:2434-2437, 1975.
52. Rodolfo M, Castelli C, Bassi C., Accomero P, Sensi M. and Parmiani G. Cytotoxic t lymphocytes recognize tumor antigens of a murine coionic carcinoma by using different T-cell receptors. Int. J. Cancer 57:440-447,1994.
53. Martinotti A, Stoppacciaro A, Vagliani M, Melani C, Spreafico F, Wysocka M, Parmiani G, Trinchieri G, and Colomob MP. CD4 T cells inhibit in vivo the CD8-mediated immune response against murine colon carcinoma cells transduced with IL-12 genes. Eur. J. Immunol. 25:137-146, 1995.
54. Brunda, MJ, Luistro L, Warrier, RR, Wright, RB, Hubbard BR, Murphy M, Wolf SF, and Gately MK. Antitumor and antimetastatic activity of interleukin 12 against murine tumors. J. Exp. Med. 178:1223-1230.1993.

APPENDIX B

HQ, USAMRMC
ATTN: MRMR-PLE
524 Palacky Street
Ft. Detrick, MD 21702-5024

DR. WILLIAM G. BRADLEY
TAMPA BAY RESEARCH INSTITUTE
10900 ROOSEVELT BOULEVARD
ST PETERSBURG, FL 33716-2308

BC990863

Di —
pl make a copy
(I.D. #) & give me back
I will see [illegible]

16/2308

ACKNOWLEDGMENT OF RECEIPT OF PROPOSAL

Your proposal to the U.S. Army Medical Research and Materiel Command DOD 1999 Breast Cancer Research Program has been received. Your proposal identification number is:

BC990863

Dissecting the Legendary Anti-Tumor Activity of a Pine Complex: Activating the Anti-Tumor Potential of Macrophage and Dendritic Cells

Please cite your proposal identification number in any further correspondence. You will not receive additional information until December 1999.

Thank you for your interest.

Kenneth A. Bertram, M.D., Ph.D., F.A.C.P.
Lieutenant Colonel, U.S. Army Medical Corps
Director, Congressionally Directed Medical Research Programs

APPENDIX C

TAMPA BAY RESEARCH INSTITUTE

Committed to the study, cure and prevention of chronic diseases.

July 21, 2003

Christopher J. Kay, Ph.D.
Patent Agent
Pendorf & Cutliff
5111 Memorial Highway
Tampa, Florida 33634-7356

Dear Dr. Kay:

This letter is to verify that the project related to Pine Cone Extract was funded during the period of January 10, 2001 through September 26, 2001 through Tampa Bay Research Institute (TBRI) Program Funds.
A foundation grant as well as TBRI Program funding covered funding prior to this time period.

Sincerely,

Diane C. Tippins

Diane C. Tippins
Business Manager

10900 Roosevelt Blvd. N. • St. Petersburg, FL 33716-2308 • (727) 576-6675 • Fax (727) 577-9862 • www.tampabayresearch.org